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Isolation of viral haemorrhagic septicaemia virus from mummichog, stickleback, striped bass and brown trout in eastern Canada

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Abstract

Viral haemorrhagic septicaemia virus (VHSV) was isolated from mortalities occurring in populations of mummichog, *Fundulus heteroclitus*, stickleback, *Gasterosteus aculeatus aculeatus*, brown trout, *Salmo trutta*, and striped bass, *Morone saxatilis*, in New Brunswick and Nova Scotia, Canada. The isolated viral strains produced a cytopathic effect on the epithelioma papillosum cyprini cell line. Serum neutralization indicated the virus was VHSV and sequencing identified the rhabdovirus isolates as the North American strain of VHSV. Phylogenetic analysis indicated that the isolates are closely related and form a distinguishable subgroup of North American type VHSV. To our knowledge, this is the first report of VHSV in mummichog and striped bass.

Keywords: brown trout, mummichog, phylogeny, stickleback, striped bass, viral haemorrhagic septicaemia virus.

Introduction

Viral haemorrhagic septicaemia (VHS) is a serious disease of fish, particularly cultured rainbow trout, *Oncorhynchus mykiss* (Walbaum), and turbot, *Psetta maximus* (L.) (Bellet 1965; Wolf 1988; Snow & Smail 1999; King, Snow, Skall & Raynard 2001; Brudeseth & Evensen 2002). The aetiological agent of VHS belongs to the *Novirhabdovirus* genus of the Rhabdoviridae (Walker, Benmansour, Dietzgen, Fang, Jackson, Kurath, Leong, Nadin-Davies, Tesh & Tordo 2000). The virus has been isolated from over 40 species of wild and farmed marine fish and from some freshwater species (reviewed in Skall, Olesen & Mellergaard 2005). The first isolation of VHS virus (VHSV) from a marine species was reported in 1979 from Atlantic cod, *Gadus morhua* L., from the Baltic Sea (Jensen, Bloch & Larsen 1979); in North America, the first detection of VHSV occurred in Washington State from adult coho, *Oncorhynchus kisutch* (Walbaum), and Chinook, *O. tshawytscha* (Walbaum), salmon in 1988 (Brunson, True & Yancey 1989). Subsequently, VHSV was isolated from several marine species in North America, including coho salmon, Pacific cod, *G. macrocephalus* (Tilesius) (reviewed in Meyers & Winton 1995), shiner perch, *Cymatogaster aggregata* (Gibbons), three-spined stickleback, *Gasterosteus aculeatus* L. (Kent, Traxler, Kieser, Richard, Dawe, Shaw, Prosperi-Porta, Ketcheson & Evelyn 1998), sardine, *Sardinops sagax* (Jenyns), mackerel, *Scomber japonicus* (Houttuyn), eulachon, *Thaleichthys pacificus* (Richardson), and surf smelt, *Hypomesus pretiosus* (Girard) (Hedrick, Batts, Yun, Traxler, Kaufman & Winton 2003). VHSV has also been isolated from farmed Atlantic salmon, *Salmo salar* L., in Spain (Jimenez de la Fuente, Marcotegui, San Juan & Basurco 1988), and in British Columbia,

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Canada (G.S. Traxler, Fisheries and Oceans Canada, personal communication). In the latter case, virus isolations usually occurred in the winter/spring period, often coinciding with herring spawning in the area; in one case chronic losses reached 10% per week. In Alaska, mass mortalities caused by VHSV were reported in Pacific herring, *Clupea pallasii* (Valenciennes), Pacific hake, *Merluccius productus* (Ayres), and walleye pollock, *Theragra chalcogramma* (Pallas) (Meyers, Short & Lipson 1999). In 1994, VHSV was isolated from asymptomatic Greenland halibut, *Reinhardtius hippoglossoides* (Walbaum), caught at the Flemish Cap, off the coast of Newfoundland, Canada (Dopazo, Bandin, Lopez-Vazquez, Lamas, Noya & Barja 2002). More recently, VHSV was isolated from muskellunge, *Esox masquinongy* (Mitchill), in Lake St-Clair, MI, USA (Elsayed, Faisal, Thomas, Whelan, Batts & Winton 2006).

With the expansion of aquaculture targeting non-salmonid marine fish species, VHSV is receiving increased attention because of its frequent isolation from a variety of marine fish species including cod, haddock, *Melanogrammus aeglefinus* L., turbot, herring, *Clupea harengus* L., flounder *Platichthys flesus* L., plaice, *Pleuronectes platessa* L., and sprat *Sprattus sprattus* L., some of which have aquaculture potential (Mortensen, Heuer, Lorenzen, Otte & Olesen 1999; Snow, Cunningham & Bricknell 2000). The reservoirs of VHSV are carrier fish among cultured, feral or wild fish in both marine and freshwater environments. Virus is shed in urine as well as coelomic fluids, whereas kidney, spleen, heart, liver and digestive tract are the sites in which virus is most abundant during clinical infection (Winton & Einer-Jensen 2002). Several factors influence VHSV susceptibility, including host species and age of fish (increased severity in younger hosts) although overt infections at all stages of the life cycle are observed (Kocan, Hershberger, Elder & Winton 2001). Genetic analysis has determined that American and European isolates of VHSV represent two distinct lineages which have been estimated to have diverged ~500–600 years ago (Basurco, Vende, Monnier, Winton, de Kinkelin & Benmansour 1995; Einer-Jensen, Ahrens, Forsberg & Lorenzen 2004). Phylogenetic analysis based on the nucleotide sequences of the partial N gene of VHSV isolates from marine and freshwater fish showed four genogroups, I to IV (Snow, Bain, Black, Taupin, Cunningham, King, Skall & Raynard 2004) and the North American isolates of VHSV from marine fish are assigned to genogroup IV.

The VHSV genome is a single linear negative-sense RNA and consists of approximately 11 200 nucleotides encoding six genes, 3′-N-P-M-G-Nv-L-5′ (Schutze, Mundt & Mettenleiter 1999). A 20-nucleotide segment in the intergenic segment between N and P genes has been shown to distinguish American and European isolates of VHSV (Einer-Jensen, Olesen, Lorenzen & Jorgensen 1995).

Through surveillance and investigations of reported mortalities of wild fish, the Department of Fisheries and Oceans Canada (DFO) has monitored the presence of various disease agents in fish across a wide geographical area in Canada. This paper reports the first detection of VHSV in four species of wild fish from the eastern Atlantic coast of Canada.

Materials and methods

Fish

Fish were frozen and transported on ice to the fish health laboratory at the Gulf Fisheries Centre where necropsy and examination were performed according to the Fish Health Protection Regulations (FHPR) Manual of Compliance (Department of Fisheries and Oceans 1984) (Table 1). The fish were examined for viruses, bacteria and parasites. No bacterial or parasitic pathogens were isolated from any of the fish reported in this study.

Virus assays

Tissues collected from the visceral organs and the gills were combined into one single pool per species. Tissues from a moribund mummichog were pooled separately. Tissue pools were screened for viral agents using the epithelioma papillosum cyprini (EPC) cell line, and two salmonid cell lines, salmonid head kidney (SHK-1) (Dannevig, Falk & Press 1995) and chinook salmon embryo (CHSE-214) cells. Briefly, tissue pools were homogenized in a 1:50 (w/v) dilution in Hanks' balanced salt solution, pH 7.6, using a stomacher (Brinkmann Instruments, Mississauga, ON, Canada). The homogenates were centrifuged using a Sorvall R2B centrifugal machine (Thermo Scientific, Whaltham, MA, USA) at 2500 ***g*** for 15 min at 4 °C and the

Table 1 Details of viral haemorrhagic septicaemia virus isolates used in the study

Isolate name	DFO case number	Species	Location	Date of collection (year/month)	Case history
CA-NB00-01	2000-149	Mummichog, *Fundulus heteroclitus* L.	Ruisseau George Collette, near Bouctouche, New Brunswick (NB)	2000/05	Five dead fish collected following a report of severe mortality in the river. One moribund fish collected.
CA-NB00-02	2000-150	Three-spined stickleback, *Gasterosteus aculeatus aculeatus* L.	Ruisseau George Collette, near Bouctouche, NB	2000/05	Five dead fish collected following a report of severe mortality in the river.
CA-NB02-01	2002-118	Striped bass, *Morone saxatilis* (Walbaum)	Miramichi Bay, Baie du Vin, NB	2002/04	Two fish, collected following a striped bass mortality event. Non-lethal lymphocystis lesions observed on skin.
CA-NB04-01	2004-81	Striped bass	Miramichi River, NB	2004/04	Three fish found dead.
CA-NS04-01	2004-175	Brown trout, *Salmo trutta trutta* L.	French River, Nova Scotia (NS)	2004/05	One sea run fish found dead.

supernatant was filtered aseptically through a 0.45-μm pore diameter membrane filter. Using the simultaneously applied cells and test sample method as described in the FHPR Manual of Compliance (Department of Fisheries and Oceans 1984), 0.1 mL of each filtrate was added in duplicate to 24-well plates (Linbro-ICN, Aurora, OH, USA) containing EPC, SHK-1 and CHSE-214 cells. Cell lines used for the assays were maintained at 15–19 °C in 75 cm^2 cell culture flasks (Corning-Costar, Acton, MA, USA) in Eagle's minimum essential medium (for CHSE-214 and EPC cells) or Leibovitz L-15 (for SHK-1 cells) containing Hanks' salts, glutamine, antibiotic-antimycotic mixture (penicillin, 1000 units mL^{-1}; streptomycin, 1 mg mL^{-1}; amphotericin B, 2.5 μg mL^{-1}) and 5% (SHK-1 cells) or 10% foetal bovine serum (FBS) (CHSE-214 and EPC cells); all media and supplements were from GIBCO-BRL (Invitrogen, Carlsbad, CA, USA). Viral cultures were incubated at 15 °C and examined for cytopathic effects (CPE) for 38 days. 10^{-1} and 10^{-3} dilutions of cell lysates from viral cultures showing CPE were subcultured onto the same cell line in which CPE was detected.

Virus neutralization

The infectious pancreatic necrosis virus (IPNV) (West Buxton) antiserum was prepared at Connaught Laboratories, Toronto, ON, Canada. Antisera against IPNV serotypes A-6 (Canada-1), A-7 (Canada-2), Canada 2/3, and antiserum against infectious haematopoietic necrosis virus (IHNV) were provided by Dr R. Kelly (retired), Department of Fisheries and Oceans, Winnipeg, MB, Canada. The anti-VHSV (F1) serum (Olesen, Lorenzen & LaPatra 1999) was provided by Dr Phil McAllister, Leetown, WV, USA.

Virus neutralization was performed for the mummichog and stickleback isolates, using the method described in the FHPR Manual of Compliance (Department of Fisheries and Oceans 1984). Briefly, 10-fold serial dilutions of a filtered (0.45 μm pore diameter) tissue culture lysate were prepared in minimum essential medium (MEM) without FBS. A 1:50 dilution of each antiserum was mixed with an equal volume of each virus dilution and incubated at 15 °C for 1 h. Duplicate wells of EPC cells grown in 24-well plates were inoculated with 0.2 mL of virus–antiserum mixture. The remaining duplicate well on each plate were inoculated with 0.1 mL of each virus dilution (without antiserum). The inoculated plates were incubated for 1 h at 15 °C to facilitate virus adsorption. Following adsorption, 1.0 mL of overlay medium (MEM-2 Hepes) was added to each well. The plates were incubated at 15 °C and observed daily for CPE. After 6 days the plates were fixed with 37% formaldehyde, stained with 1% crystal violet alcohol and the results recorded.

RT-PCR

For the confirmation of VHSV in the stickleback and mummichog samples, RNA was extracted from cell cultures using the Trizol LS method (Life Technologies, Gaithersburg, MD, USA). The resulting RNA

Table 2 Primers used for reverse transcription-polymerase chain reaction and sequencing viral haemorrhagic septicaemia virus

Primer	Position[a]	Sequence 5′ → 3′	Reference
VHS 3	2965–2987	CGGCCAGCTCAACTCAGGTGTCC	Williams, Blake, Sweeney, Singer & Nicholson (1999)
VHS 4	3589–3564	CCAGGTCGGTCCTGATCCATTCTGTC	Williams *et al.* (1999)
P1	430–452	GACAAGATGATCAAGTACATCAC	Einer-Jensen *et al.* (1995)
P2	1153–1134	TTCCGGTGGAGCTCCTGAAG	Einer-Jensen *et al.* (1995)
P3	1323–1305[a]	TGTTGTGTGGAACAGCCAGTG	Einer-Jensen *et al.* (1995)
P4	1279–1256	AGAGAAAAATTCTTATAATCGTGCC	This study
842F	762–782	GTCAGCCAGGCGGATCATCCA	This study, for N gene sequencing
956R	869–849	CTTGTAGTAGGACTCTCCCAGC	This study, for N gene sequencing
3485F	3485–3505	ATCACAGGGTGGTCAAGGCAA	This study
3809R	3809–3789	CTGGGACGAAACTTTGAGAGGAG	This study
2852F	2852–2874	CACAGATCACTCAACGACCTCCG	This study
3571R	3571–3550	ATTCTGTCCCGCAAAATGTCAC	This study

[a]Based on accession no. Z93414, cod ulcus sequence, except primer P3 (from X59241, Makah).

pellet was suspended in 20 μL of DEPC-treated water. Reverse transcription-polymerase chain reaction (RT-PCR) was conducted with the Ready-To-Go system (Amersham Pharmacia Biotech, Uppsala, Sweden). Briefly, 4 μL of resuspended RNA was reverse transcribed with random hexamers (2.5 μg) in a total volume of 40 μL. The mixture was incubated at 42 °C for 30 min, 95 °C for 5 min, and left at 4 °C until primers were added for the PCR reaction. A total volume of 50 μL was used in PCR. Final concentrations of primers and $MgCl_2$ were 0.8 μM and 2 mM, respectively. Primers initially used were VHS 3 and VHS 4 (approximately 625 bp product), designed from conserved G gene sequences (Table 2). Subsequently, two sets of primers, P1 and P2 (approximately 724 bp product) and P1 and P3 (approximately 803 bp product) were used. These primer sets were designed to distinguish European from American isolates through the amplification of nucleoprotein (N) gene fragments, with P1 and P2 being complementary to all tested isolates and P3 being complementary only to a North American isolate (Einer-Jensen *et al.* 1995). Amplification conditions consisted of 35 cycles of 94 °C for 60 s, 60 °C for 60 s and 72 °C for 1 min 30 s, followed by a 7-min extension period at 72 °C. All amplifications were conducted using a GeneAmp 9600 (PE Applied Biosystems, Foster City, CA, USA). RT-PCR products were mixed with non-denaturing loading buffer and visualized by running 5 μL on 11% acrylamide Tris-borate EDTA (TBE) mini gels and staining with ethidium bromide.

For the other samples, RNA was extracted from cell lysates with TRI Reagent™ LS (Molecular Research Center, Inc., Cincinnati, OH, USA). Total RNA samples were redissolved in 20 μL each of sterile H_2O containing RNase inhibitor. Reverse transcription primed with random hexamers was carried out with a RevertAid™ First Strand cDNA synthesis kit (MBI Fermentas Inc., Burlington, ON, Canada) using up to 5 μg of RNA and following the manufacturer's instructions except that RNA was denatured at 95 °C for 5 min. PCR amplifications were carried out with AmpliTaq Gold® PCR Master Mix (Applied Biosystems) in 25-μL volumes containing 2 μL of cDNA, 0.4 μM of each primer, 1 μL of bovine serum albumin 1% and 2.5 mM $MgCl_2$. PCR conditions were 94 °C for 4 min (initial denaturation), followed by 10 cycles of 94 °C for 30 s, 60 °C (−1 °C per cycle) for 30 s, 72 °C for 90 s and then 35 cycles of 94 °C for 30 s, 50 °C for 30 s, 72 °C for 90 s, and a final hold at 72 °C for 5 min. Amplifications were conducted using an Eppendorf Mastercycler (Brinkmann Instruments). Amplification products were resolved on 1.5% agarose gels stained with ethidium bromide. Primers used for the confirmation of VHSV presence were 3485F and 3809R from the glycoprotein (G) gene, and P1/P4 were used for amplification of the 3′ terminal portion of the N gene. Additional primers were used for complete resolution of sequences (Table 2). For the amplification of a partial G gene segment for sequencing, primers 2852F and 3571R were used (Table 2).

Sequencing

The P1/P4 (N gene) and 2852F/3571R (G gene) PCR products were purified directly using Microcon-PCR filter units (Millipore) or after extraction from gels using Spin-X centrifuge tube filters (Corning). The purified amplification products

Table 3 Isolates used for phylogenetic comparison of viral haemorrhagic septicaemia virus isolates

Virus isolate	Country of origin	Year of isolation	Accession no.	Description	Genogroup[a]	Nt/aa identities (N gene)[b]	Nt/aa identities (G gene)[c]
CA-NB00-01	Canada, Atlantic	2000	EF079895 EF079896	Mummichog	IVb	–	–
CA-NB02-01	Canada, Atlantic	2002	EF079897	Striped bass	IVb	100/100	99.5/99.5
CA-NB04-01	Canada, Atlantic	2004	EF079898	Striped bass	IVb	100/100	99.7/100
CA-NS04-01	Canada, Atlantic	2004	EF079899	Brown trout	IVb	100/100	99.7/100
MI04	USA, Michigan	2004	DQ401193	Muskellunge	IVb	n/a	97.2/98.6
MI03GL	USA, Michigan	2003	DQ427105	Muskellunge	IVb	98.8/99.6	n/a
ME03	USA, Maine	2003	DQ401192	Atlantic herring	IVa	n/a	95.4/96.7
BC93-372	Canada, Pacific	1993	DQ401186	Pacific herring	IVa	n/a	95.4/96.7
BC99-001	Canada, Pacific	1999	DQ401195	Pacific sardine	IVa	n/a	95.2/96.7
BC99-010	Canada, Pacific	1999	DQ401194	Pacific herring	IVa	n/a	95.2/96.7
BC99-292	Canada, Pacific	1999	DQ401188	Atlantic salmon	IVa	n/a	95.4/96.3
BC98-250	Canada, Pacific	1998	DQ410187	Atlantic salmon	IVa	n/a	95.2/96.3
US-Makah	USA, Washington	1988	X59241 U28747	Coho salmon	IVa	94.4/94.4	95.4/96.7
JP-KRRV9822	Japan	1998	AB179621	Japanese flounder, farmed *Paralichthys olivaceus*	IVa	94.6/98.4	94.8/96.3
Japan99	Japan	1999	DQ401191	Japanese flounder	IVa	n/a	94.6/96.3
FR-14-58	France	1990	AF143863	Rainbow trout	Ia	87.4/96.4	85.5/95.8
FR-07-71	France	1971	AJ233396	Rainbow trout	Ia	87.5/95.6	85.4/94.9
DE-Fil3-wt	Germany	1983	X73873	Rainbow trout, Wild type isolate	Ia	87.8/96.4	85.8/94.9
DE-Fil3	Germany	1983	Y18263	Rainbow trout	Ia	87.8/96.4	85.8/94.9
DK-Hededam	Denmark, Hededam	1972	Z93412	Rainbow trout	I	87.8/96.0	86.8/95.3
DK-codulcus	Denmark, North Sea	1979	Z93414	Atlantic cod	Ib	87.5/96.8	86.0/95.8
UK-96-43	English Channel	1996	AF143862	Atlantic herring	Ib	87.3/96.0	85.8/94.9
DK-1p52	Denmark, Baltic Sea	1996	AY546576	Sprat *Sprattus*	II	n/a	85.5/93.9
DK-1p53	Denmark, Baltic Sea	1996	AY546577	Atlantic herring	II	n/a	85.5/93.9
UK-H17/5/93	Shetland, North Sea	1993	AY546630	Atlantic cod	III	n/a	86.1/95.8
DK-4p101	Denmark, North Sea	1997	AY546581	Whiting *Merlangius merlangus*	III	n/a	85.2/94.4
DK-4p168	Denmark, Skagerrak	1997	AY546582	Atlantic herring	III	n/a	86.4/96.3

[a]Genogroups according to Einer-Jensen *et al.* (2004), Snow *et al.* (2004) and this study.
[b]Identities with CA-NB00-01 sequence; length for calculation of identities in the partial N gene are 818 nucleotides (nt) and 251 amino acids (aa).
[c]Identities with CA-NB00-01 sequence; length for calculation of identities in the partial G gene are 649 nucleotides (nt) and 214 amino acids (aa).

were sequenced using PCR primers P1, P4, 842F, 956R (for the N gene) and 2852F, 3571R (for the G gene), respectively, on both strands of the fragments. Sequencing was performed on an ALFexpress II using Thermo Sequanase Cy5 dye terminators (Amersham Biosciences, GE Healthcare, Baie d'Urfe, QC, Canada) or samples were sent to Northwoods DNA Inc. (Solway, MN, USA) for sequencing.

Phylogenetic analysis

Sequences were assembled using GeneDoc (http://www.psc.edu/biomed/genedoc), and primer sequences were removed. A BLAST similarity search (Altschul, Gish, Miller, Myers & Lipman 1990) for significant homologous sequences was performed against GenBank (National Center for Biotechnology Information) using the partial sequence of the VHSV nucleoprotein and glycoprotein gene. Homologous sequences were retrieved from GenBank for phylogenetic analysis and are listed in Table 3. Selected sequences were used for the phylogenetic analysis of the partial G gene sequence (Table 3).

Multiple alignments of nucleotide sequences were performed using ClustalX (1.81) (Thompson, Gibson, Plewniak, Jeanmougin & Higgins 1997). Default parameters of multiple alignments were used and results were inspected visually. Aligned sequences were trimmed at their ends. Phylogenetic analysis was conducted using maximum likelihood analysis and TREE-Puzzle (5.2) (Schmidt, Strimmer, Vingron & von Haeseler 2002). TreeView version 1.6.6 (Page 1996) was used to view the trees.

Results

Necropsy observations

All specimens were dead at the time of collection with exception of one mummichog which was moribund and noted to have ecchymotic haemorrhage of the submandibular dermis. The moribund mummichog presented with diffuse dermal haemorrhaging of the skin ventrally, and a distended abdomen containing a copious amount of sanguinous fluid in the abdominal cavity. No signs of disease were noted for any of the other fish examined.

Viral isolation

A CPE consisting of retracted and rounded refractile cells was noted for all pools cultured on the EPC cell line between days 8 and 13. Following subculture, extensive CPE was observed after 4 days at both the 10^{-1} and 10^{-3} dilutions of the harvested cell lysates. No CPE was detected in the CHSE-214 and SHK-1 cell lines after 38 days of incubation during the initial assay for all samples except for the striped bass samples received in 2002, which also tested positive for CPE on CHSE cells.

Virus neutralization

The initial attempt to identify the virus isolated from mummichog and stickleback using antiserum against IPNV (WB) failed to produce any evidence of antigenic similarity. Because IPNV is enzootic in the Maritime region of eastern Canada, it was thought that the isolate may be one of the other prevalent serotypes; hence, neutralization testing using antisera against the other known serotypes (Can-1, Can-2 and Can-2/3) was conducted. Again, antigenic relatedness was not evident. The virus was then tested using antisera against IHNV and VHSV (F1). Neutralization test results are shown in Table 4. The mummichog virus was completely neutralized by the VHSV antiserum. The virus was not neutralized by the IHNV antiserum.

RT-PCR

Primers VHS 3 and VHS 4 produced an approximately 625 bp product identifying the stickleback and mummichog isolates as VHSV. Subsequent analysis with the P1 and P2 primers produced the expected size product as did the P1 and P3 primers, confirming their identity as North American strains. Subsequent isolates were tested with primers 3485F/3809R and produced the expected product, confirming their identity as VHSV (results not shown).

Table 4 Serum neutralization of mummichog virus isolate against VHSV (F1), IHNV and IPNV antisera

	Virus dilutions				
Antiserum (1:50)	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Anti-VHSV (F1)	+	+	+	+	+
Anti-IHNV	–	–	–	–	–
Anti-IPNV (WB)	–	–	–	–	–
Anti-IPNV (Can-1)	–	–	–	–	–
Anti-IPNV (Can-2)	–	–	–	–	–
Anti-IPNV (Can 2/3)	–	–	–	–	–

+, neutralization; –, no neutralization.

Sequencing

Sequencing of the partial nucleoprotein (N) gene using P1/P4 RT-PCR products was performed on both strands, using internal primers to completely resolve the fragment. The stickleback, mummichog, striped bass and brown trout isolates were identical for that fragment, however the stickleback fragment was sequenced from P1 to P3; thus this sequence was not used for phylogenetic analysis. BLAST search algorithm (Altschul *et al.* 1990) analysis of 818 bp of the partial N gene showed that the isolates had highest nucleotide homology to the Muskellunge isolate MI04, the Japanese flounder isolate KRRV9822 and the North American Makah VHSV isolated from coho salmon with 99%, 95% and 94% homology, respectively. Similarity was also seen with VHSV isolates from other types of fish such as rainbow trout (isolates DK-Hededam and FR-14-58), cod (isolate DK-cod ulcus), and herring (isolate UK-96-43), the homology being 87–88% with all of these isolates when 818 bp of the N gene were compared (Table 3). Comparison of amino acids in the third open reading frame of the sequence (excluding the non-coding region) showed that the MI04 isolate had more than 99% homology to CA-NB00-01, 94% homology with the Makah isolate compared with 96–97% homology with the European isolates and 98% homology with the Japanese flounder isolate (Fig. 1 and Table 3).

The partial nucleoprotein sequence we chose encompasses the 20 nucleotides specific to North

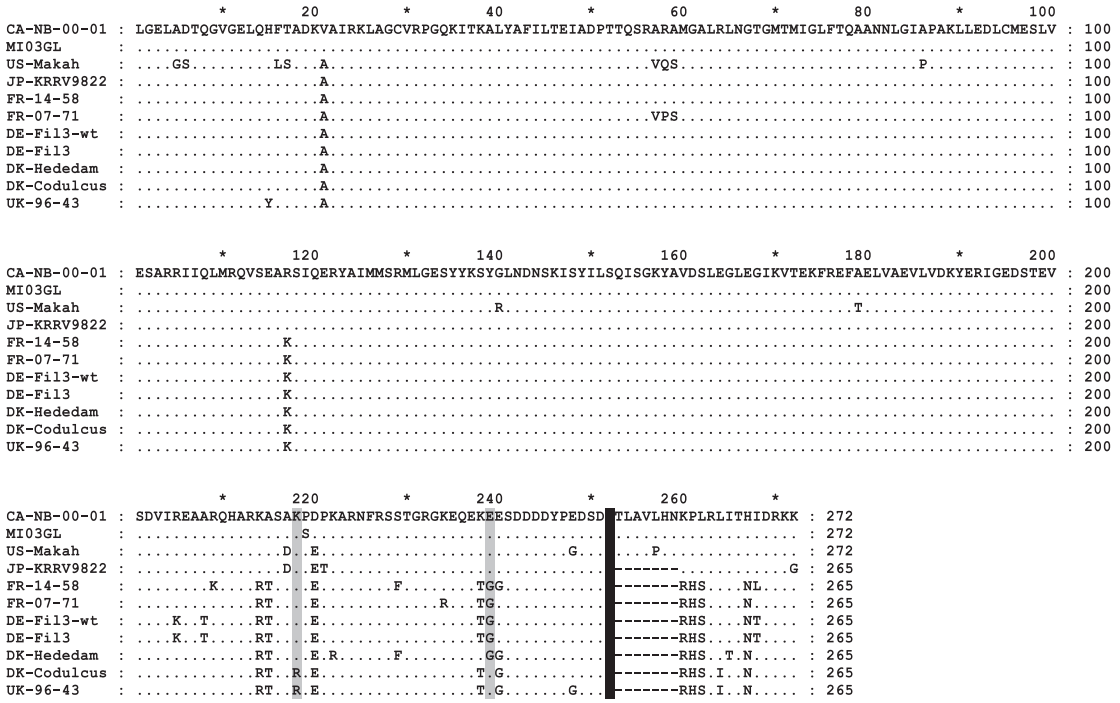

Figure 1 Partial nucleoprotein gene (N) amino acid alignment of viral haemorrhagic septicaemia virus isolates. Stop codon (X) is shaded (black) and 3′ non-coding portion of the sequence is left for comparison. Shaded (grey) amino acids (pos 371 and 392 of the full N gene) are referred to in Discussion.

American strains, and deleted in European isolates (Einer-Jensen *et al.* 1995). Compared with the US-Makah isolate, Atlantic Canadian isolates and the MI04 isolate have two identical nucleotide substitutions in this segment.

Sequencing of the partial glycoprotein (G) gene product was performed for all Atlantic Canadian isolates except CA-NB00-02 (stickleback) which did not amplify well by PCR after prolonged freezing. The isolates were highly homologous with two to three nucleotide differences over the 649 bp sequence and one amino acid difference. The North American MI04 had highest homology to the eastern Atlantic group, with 97% nucleotide similarity and 98% amino acid similarity. Partial N and G gene sequences of the Canadian isolates were submitted to GenBank (accession nos. EF079895–EF079899).

Phylogenetic analysis

Maximum likelihood analyses using the nucleotide sequences of the partial glycoprotein (G) gene were conducted to infer the phylogenetic relationship of VHSV isolates from Atlantic Canada. We used the arbitrary nomenclature suggested by Einer-Jensen *et al.* (2004) and Snow *et al.* (2004) to define VHSV genogroups.

The relationship among the four Canadian isolates and 22 selected sequences is illustrated in a phylogenetic tree (Fig. 2), in which the genotypes I to IV were well supported. The previous grouping obtained by Einer-Jensen *et al.* (2004) and by Snow *et al.* (2004) was confirmed. Genogroup IV comprising North American isolates was separated in two sublineages. Subgroup IVa is assigned to the US-Makah and Japanese flounder isolates and subgroup IVb comprised Atlantic Canadian isolates, this latter group branching earlier than the isolates from the Pacific. Further separation of Atlantic Canadian isolates from the MI04 isolate is suggested in the phylogenetic tree using the partial G gene sequence. However, a similar tree produced with the N gene sequences grouped these isolates (not shown).

Discussion

In this work, we describe the isolation and characterization of VHSV from mummichog, stickleback, striped bass and wild brown trout. The mummichog and stickleback isolates were recovered from

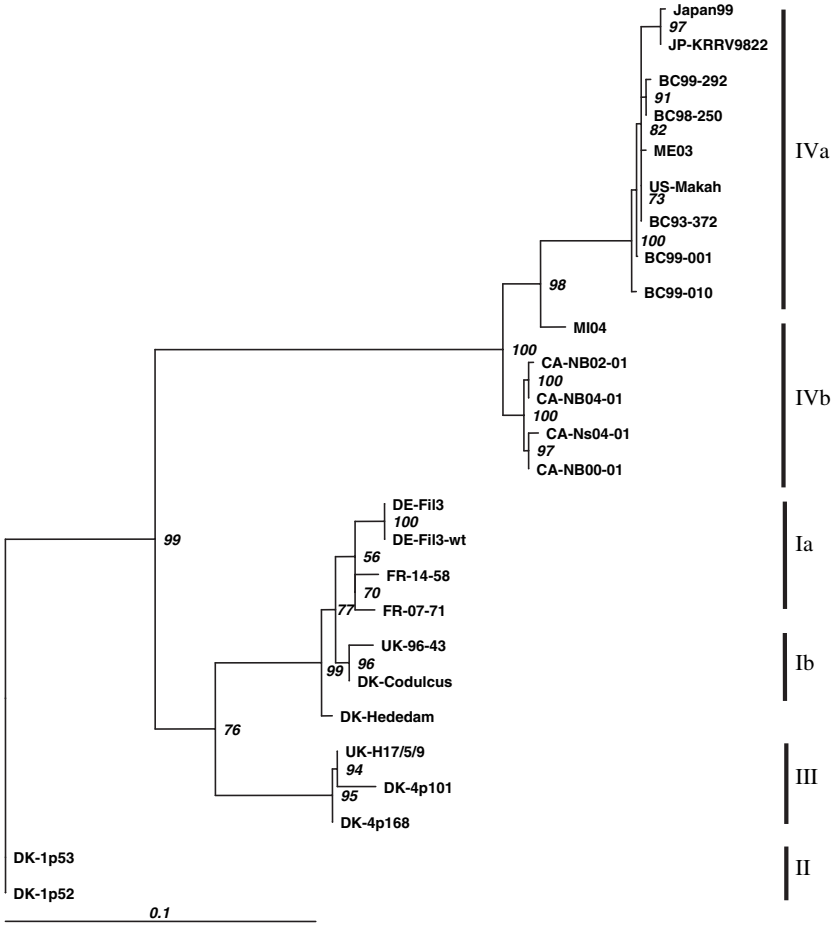

Figure 2 Consensus tree for viral haemorrhagic septicaemia virus isolates constructed using Tree-Puzzle 5.0 and maximum likelihood method, and inferred with 1000 quartet puzzling steps and maximum likelihood branch lengths. Phylogram generated using partial G gene sequences, indicating the four major genotypes. Dk-1p52 was used as outgroup. The scale bar indicates the number of substitutions per nucleotide site.

fish in the same area, following a report of thousands of dead fish. Although no other significant pathogens were recovered from the mummichogs and sticklebacks, there is insufficient evidence to conclude that VHSV was responsible for these mortalities, as well as those of the other three affected species, as the fish samples submitted were not suitable for histological examination and other factors that could have contributed to this event were not investigated. CPE was only observed in the EPC cells for all samples tested, and VHSV was identified by serum neutralization with the F1 antisera. The CHSE-214 cell line is susceptible to VHSV, although to a lesser extent (Hedrick *et al.* 2003) and only one submission of striped bass produced CPE in this cell line during the initial assay. The lower sensitivity of CHSE-214 cells to VHSV could be due to phenotypic differences between isolates, inherent sensitivity, and/or cell culture conditions.

To our knowledge, this is the first report of VHSV in mummichog and striped bass. VHSV has been isolated from asymptomatic sticklebacks in British Columbia, Canada (Kent *et al.* 1998). An epizootic caused by VHSV was described in cultured brown trout (de Kinkelin & le Berre 1977) and in a population of wild brown trout in Germany (Enzmann, Konrad & Rapp 1992). The isolation of VHSV from new host species in Atlantic Canada could indicate the spread of the virus but we suspect the virus to have been present but undetected. The surveillance programme and systematic screening for VHSV, and other salmonid viruses, began in 1977 on the East coast of Canada.

The programme focuses predominantly on salmonids; however, since 1997 testing of cultured and wild non-salmonid fish species has increased.

Successful amplification of part of the nucleoprotein (N) gene using P1 and P3 primers typed the mummichog and stickleback isolates as North American, as it has been shown that an American isolate contains a unique 20 nucleotide sequence to which the P3 primer can anneal (Einer-Jensen *et al.* 1995). All the isolates were identical in the region covered by primers P1 and P4. All the isolates had two mutations in the 20 nucleotide segment specific to North American isolates, which is located immediately at the end of the stop codon for the N gene, and their closest relative, the MI03GL isolate possesses the same mutations. Interestingly, the deduced amino acid sequence of the US-Makah isolate was more divergent from the Atlantic Canadian isolates for that gene than the European isolates, with numerous non-silent nucleotide differences. The Japanese flounder isolate JP-KRRV9822 has a high nucleotide and amino acid homology to the Atlantic Canadian isolate when comparing the N gene sequences. However, the Japanese flounder isolate possess the 20 nucleotide deletion characteristic of European isolates. This suggests that the intron 3′ of the N gene is a hot spot for deletion. Further examination of the aligments obtained did not reveal any significant mutation trends, especially between virulent and avirulent isolates. Mutations identified previously (Betts & Stone 2000) as potential determinants of virulence were examined. For the N gene, the glutamic acid at position 392 (Fig. 1) was present in the North Atlantic isolates whereas isolates virulent for trout have a glycine substitution. However, lysine at position 371 of the N gene is conserved between North American and isolates DK-Hededam and FR-14-58 which are virulent for trout.

The homology of sequences found in the isolates, although spanning a number of years and host species, supports the notion of a lower evolution rate for marine VHSV, compared with virus from farmed freshwater fish (Einer-Jensen *et al.* 2004). VHSVs have a wide host range and spatial distribution. Although VHSV has been present on the West coast of Canada for several years (Kent *et al.* 1998; Meyers *et al.* 1999; Hedrick *et al.* 2003), there had been no indication of its presence on the East coast, except for its isolation from asymptomatic Greenland halibut caught at the Flemish Cap, a deep fishing ground in international waters near Newfoundland (Dopazo *et al.* 2002). However, this population is believed to migrate from Norway, the Svalbard Islands and Iceland to the Grand Banks and is unlikely to have been in direct contact with the fish studied in this work (Bowering & Atkinson 2003). Furthermore, the Greenland halibut VHSV isolate belongs to genotype III (Einer-Jensen, Winton & Lorenzen 2005) which comprises mainly isolates from the North Sea and waters around the UK.

On the Pacific coast, the distribution of VHSV isolates indicates that migratory mixing of populations have occurred between Canada and USA, and as far as Japan; an exception being the isolation of a European genotype of VHSV found in Japan suspected to have been introduced accidentally (Nishizawa, Iida, Takano, Isshiki, Nakajima & Muroga 2002). The close homology between the Lake St-Clair (Michigan), West and East coast Canadian isolates of VHSV suggests a recent common ancestry between these three groups. The presence of closely related yet distinct strains of VHSV on both coasts could be the result of viral dispersal via unknown vectors. It also suggests that no recent mixing has occurred in the North Atlantic between Canadian and European waters. A different situation is observed for nodaviruses, where we have observed a closer homology between isolates from North Atlantic eastern Canada, and northern regions of Europe, e.g. Norway (Gagne, Johnson, Cook-Versloot, MacKinnon & Olivier 2004).

Considering that RNA viruses are prone to mutate, and the lack of detailed knowledge of the molecular basis of VHSV pathogenicity, one cannot ignore the potential threat marine strains of VHSV pose to the fish-farming industry. The impact of some European isolates of VHSV on early life stages of susceptible species (i.e. rainbow trout and turbot) is known (Wolf 1988); however, the impact of the other genotypes on these and other species is not well documented. Recent studies have shown that Atlantic cod and Atlantic halibut were not susceptible by immersion challenge to most VHSV genotypes, including the US-pws-ak90 isolate (genotype IVa), whereas turbot was susceptible to isolates from genotypes Ib and III but not to the USA-pws-ak90 isolate (Snow, King, Garden & Raynard 2005a; Snow, King, Garden, Shanks & Raynard 2005b). Similarly, chronic infection of older fish and impairments caused by VHSV infection combined with other stressors and cold

water temperatures favouring infection (Castric & de Kinkelin 1984) could be cause for concern if the virulence of these strains for local eastern Canadian host species is confirmed.

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